MINIREVIEW

Black holes, antivirulence genes, and gene inactivation in the evolution of bacterial pathogens

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Abstract

The evolution of bacterial pathogens from nonpathogenic ancestors is marked principally by the acquisition of virulence gene clusters on plasmids and pathogenicity islands via horizontal gene transfer. The flip side of this evolutionary force is the equally important adaptation of the newly minted pathogen to its new host niche. Pathoadaptive mutations take the form of modification of gene expression such that the pathogen is better fit to survive within the new niche. This mini-review describes the concept of pathoadaptation by loss of gene function. In this process, genes that are no longer compatible with the novel lifestyle of the pathogen are selectively inactivated either by point mutation, insertion, or deletion. These genes are called 'antivirulence genes'. Selective pressure sometimes leads to the deletion of large regions of the genome that contain antivirulence genes generating 'black holes' in the pathogen genome. Inactivation of antivirulence genes leads to a pathogen that is highly adapted to its host niche. Identification of antivirulence genes for a particular pathogen can lead to a better understanding of how it became a pathogen and the types of genetic traits that need to be silenced in order for the pathogen to colonize its new host niche successfully.

Introduction

All living organisms evolve. As multicellular organisms, plants, animals, and humans evolved on earth, bacteria, viruses, and other microorganisms also adapted and evolved to populate the new niches provided by these larger organisms. While some microorganisms evolved a parasitic but benign lifestyle in their multicellular hosts, others evolved a more aggressive and pathogenic lifestyle that ultimately harms or even kills the host. The fate of these microorganisms, which we classify as microbial pathogens, is dictated by their ability to grow within the host and be transmitted to a new host(s) before the initial host succumbs or mounts a sterilizing immune response against the invader.

The evolution of bacterial pathogens is similar to the evolution of any other organism. Mutations occur due to mistakes in DNA replication or mutagenic conditions in the environment and those mutations that benefit the organism are maintained and passed on to succeeding generations of progeny. Selective forces in the environment determine the winners and losers in the genetic lottery. But bacteria, with their short generation time and the ability to grow to hundreds of billions of individuals, have a decided advantage in this game. Moreover, bacteria need not depend solely on the occasional random mutation to improve their fitness but have evolved mechanisms for acquiring genes directly from other bacteria through contact-mediated transfer of DNA (conjugation) and indirectly via bacteriophage vectors (transduction) or the uptake of naked DNA from their surroundings (transformation). These forms of horizontal gene transfer (HGT) vastly expand a bacterium's potential for genetic adaptation (Ochman *et al.*, 2000). Thus, HGT provides a bacterium with the ability to acquire genes that have already proved their usefulness (i.e. have survived selective pressures) in other bacteria.

The past decade has witnessed an explosion of knowledge of the genetic nature of bacterial pathogens thanks largely to the application of whole genome sequencing. The genome of every major bacterial pathogen of human and veterinary importance has now been sequenced (links to Complete Microbial Sequences can be found at http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). In addition, the genomes of

many nonpathogenic bacteria (environmental saprophytes and commensals of human) have been sequenced. These data provide an unprecedented opportunity to examine how bacterial pathogens evolved from their commensal ancestors.

A well-established theme in the evolution of bacterial pathogens is acquisition of novel gene traits through HGT. One of the earliest demonstrations of how plasmids contribute to bacterial virulence came from the seminal work of H. Willy Smith and his colleagues, who showed a role for plasmids in bacteria that cause diarrhea in piglets (Smith & Linggood, 1971). In the ensuing years, many more examples of plasmids that encode virulence determinants that allow pathogenic bacteria to colonize new niches and cause damage to the host have been described. Bacteriophage genomes, too, are sources of genes that encode traits that allow the newly evolved pathogen to colonize and compete successfully within a new niche (Brussow *et al*., 2004).

Of considerable impact to the evolution of bacterial pathogens is the contribution made by pathogenicity islands (PAI). A PAI is a large block of genes (10–200 kb) present in a bacterial pathogen that is missing from a related but nonpathogenic reference strain (Hacker & Kaper, 2000; Schmidt & Hensel, 2004). The island contains genes that are known or suspected to play a role in the virulence of the pathogen. Furthermore, the codon usage of the genes in the island and the atypical G+C content of the DNA in the island relative to the reference genome suggest their foreign origin and acquisition by HGT. Often, one or more PAIs can be identified within the genome of a bacterial pathogen, suggesting that a bacterium can evolve into a pathogen by quantum leaps, i.e. acquisition by HGT of a cluster of virulence genes in the form of a PAI in a single step (Groisman & Ochman, 1996).

While there are numerous examples of pathogen evolution by gene acquisition by plasmids, bacteriophages, and PAIs, the contribution of gene loss to pathogen evolution is only now beginning to be appreciated. This mini-review will discuss pathoadaptation and how loss of gene function contributes to pathogen fitness.

Pathoadaptation

Although pathogen evolution progresses through mutation and gene acquisition via HGT, the newly acquired virulence traits, along with the pre-existing core genes, continue to undergo selection after the pathogen has acquired the ability to colonize a new niche. This process is known as pathoadaptation. Pathoadaptive mutations are genetic modifications that enhance the fitness of the pathogen in the novel (host-associated) environment. One example of pathoadaptation is at the level of virulence gene expression. In many pathogens, newly acquired virulence genes, whether present on a plasmid or a PAI, appear to have been brought under control of a regulator that was already present in the core genome of the pathogen's ancestor. In *Shigella* spp., expression of the genes encoding invasion effectors and the type III secretion system (T3SS) that exports these effectors is tightly regulated by growth temperature (Dorman & Porter, 1998). The genes are expressed at 37 °C, the temperature of the human host, and repressed at lower temperatures (Maurelli *et al*., 1984). This temperature regulation is controlled by two transcriptional activators, VirF and VirB, encoded on the virulence plasmid of *Shigella* and one of these activators is in turn controlled by a chromosomally encoded repressor, H-NS (Maurelli & Sansonetti, 1988). The gene for H-NS is conserved in just about all *Enterobacteriaceae*, pathogens, and commensals, and is clearly not specifically a virulence gene (Bertin *et al*., 1999). The 'hard wiring' of plasmid-encoded virulence genes of *Shigella* into a pre-existing regulatory circuit is an adaptation to regulate virulence gene expression such that it occurs only when the pathogen is inside its host. In this way, *Shigella* conserves energy by not synthesizing a complex surface structure, the T3SS, for invading mammalian cells when it is not within the mammalian host.

Genetic variation of the FimH adhesion of type 1 fimbriae expressed by uropathogenic *Escherichia coli* is another example of pathoadaptive mutation. While $> 98\%$ of *E. coli* isolates express the common type 1 pili, uropathogenic isolates express a variant form of FimH. The variant adhesion shows increased binding to mannosylated glycoproteins and confers a significant advantage for colonization of the bladder compared with the adhesion found in commensal strains that colonize the intestine (Sokurenko *et al*., 1998). However, the variant FimH is more sensitive to soluble inhibitors present in the oropharyngeal mucosa and may be detrimental for transmission. In this case, the pathoadaptative mutation that results in increased virulence comes at the expense of decreased commensal fitness of the microbe in the intestine.

Pathogenic bacteria also evolve from commensal bacteria by 'loss of function'. Thus, pathoadaptive mutation via gene loss complements the pathway of bacterial pathogen evolution by 'gain of function' mutation and gene acquisition. This model of evolution begins with the premise that genes required for fitness in one niche may actually inhibit fitness in another environment. For example, a bacterium living as a commensal within a certain niche in the host is subject to selective pressures that result in the evolution of an organism that colonizes and makes optimal use of the available nutrients in that niche. If the bacterium acquires new genes that allow it to colonize a new niche, a new set of selective pressures will be brought to bear within the new niche. These forces will favor the emergence of variant strains of the pathogen that have eliminated or down-regulated

expression of any gene that is incompatible with growth in the new niche. When the gene in question affects the pathogenicity of the organism, it is called an 'antivirulence' gene. Therefore, we define an 'antivirulence' gene as a gene whose expression in a bacterial pathogen is incompatible with the virulence of that pathogen. Each newly evolved pathogen adapts to its new lifestyle by eliminating or reducing expression of the antivirulence gene in its genome. Adaptation can be achieved by deletion of the antivirulence gene, generating 'black holes' in the pathogen genome. It can also be accomplished by point mutations within the gene or suppression of gene expression. Pathogen variants that have undergone this sort of genetic 'fine-tuning' to adjust to the new lifestyle in the host niche are now better fit to populate that niche. Thus, these variants tend to be favored by genetic selection and give rise to present-day pathogens.

It is important to make a distinction between pathoadaptative mutation by loss of antivirulence genes and reductive evolution. In the latter process, commitment to an obligate intracellular lifestyle for certain pathogens results in loss of genes not essential to life within the host (Moran & Plague, 2004). Examples of genome reduction include *Mycobacterium leprae* (Cole *et al*., 2001), *Coxiella burnetii* (Seshadri *et al*., 2003), and the Rickettsiae (Andersson *et al*., 1998). Not only have these organisms deleted genes that are no longer required for their survival but their restricted lifestyle inside a host cell limits opportunities for gene acquisition from other microorganisms and contributes to their reduced genome size. In practice, it is not easy to distinguish loss of antivirulence genes from genome reduction. It is not intuitively obvious that a gene has been lost because it has antivirulence properties as opposed to being superfluous for survival in a new niche. Therefore, an experimental test of the antivirulence phenotype of a given gene is required for defining an antivirulence gene.

cadA – the black hole in *Shigella flexneri*

The best example of pathoadaptive mutation by loss of antivirulence genes is the case of the *cadA* gene in *Shigella* spp. Bacteria of the genus *Shigella* are gram-negative rods that are the causative agents of bacillary dysentery or shigellosis. The bacteria are highly host adapted and cause disease only in humans and primates. *Shigella* invade cells of the colonic epithelium, replicate intracellularly, and spread from cell to cell, causing abscesses and ulcerations of the intestinal lining and leading to the bloody mucoid stools characteristic of dysentery. Bacterial invasion and replication is accompanied by an intense inflammatory response that benefits both the host and the pathogen (Sansonetti *et al*., 1999).

The four species of *Shigella* (*Shigella dysenteriae*, *S. flexneri*, *Shigella boydii*, and *Shigella sonnei*) are so closely related to *E. coli* that they should be included in a single species. In fact, their genomes are colinear and more than 90% homologous (Jin *et al*., 2002). However, studies have revealed that *Shigella* strains do not form a single subgroup of *E. coli*, as would be expected of a distinct genus, but are derived from separate *E. coli* lineages (Pupo *et al*., 1997; Pupo *et al*., 2000). Acquisition of the large virulence plasmid was the crucial event in the evolution of *Shigella* from the nonpathogenic commensal *E. coli*. The plasmid is found in all species of *Shigella* and encodes genes for expression of the hallmarks of *Shigella* virulence: invasion, intracellular replication, intercellular spread, and induction of an inflammatory response. The clustering of these virulence genes and their low G+C content relative to the rest of the plasmid genes also suggest that they constitute a PAI within the plasmid. Thus, horizontal transfer of the virulence plasmid from an unknown donor to commensal *E. coli* occurred multiple times, on each occasion giving rise to a new *Shigella* clone (Pupo *et al*., 2000).

In addition to acquisition of the virulence plasmid, *Shigella* also acquired PAIs. For example, *S. flexneri* contains two PAIs. The 46 kb SHI-1 encodes the ShET1 enterotoxin as well as a cytopathic protease and a mucinase (Rajakumar *et al*., 1997; Al-Hasani *et al*., 2001). SHI-2 encodes an aerobactin iron transport system and immunity to several colicins (Moss *et al*., 1999; Vokes *et al*., 1999).

As outlined above, ancestral traits that are incompatible with virulence eventually are lost from the newly evolved pathogen genome as increased fitness favors growth of the pathoadapted clones carrying these beneficial mutations. Therefore, traits absent in all pathogenic clones of a species, but uniformly expressed in the closely related nonpathogenic ancestor species, are strong candidates for pathoadaptive mutations that have arisen by convergent evolution. Evidence in support of this new model of pathogen evolution was first provided by comparison of *Shigella* with its commensal ancestor *E. coli*. While *Shigella* and *E. coli* share many biochemical traits, some properties clearly differentiate *Shigella* from *E. coli*. One of these is lysine decarboxylase (LDC) activity, which is encoded by the *cadA* gene in *E. coli*. Whereas LDC is expressed in $> 90\%$ of *E. coli* isolates, no strains of *Shigella* express LDC activity. Moreover, enteroinvasive strains of *E. coli* that cause a disease similar to dysentery caused by *Shigella* also lack LDC activity (Silva *et al*., 1980). The absence of LDC activity in the Shigellae suggested that *cadA* may be an antivirulence gene for *Shigella*. Experimental evidence for the antivirulence nature of the *cadA* gene came from measurement of the virulence phenotypes of a strain of *S. flexneri* 2a that was transformed with the *cadA* gene from *E. coli* K-12 (Table 1). The LDC-producing *Shigella* is still invasive but it fails to produce the

wild-type level of enterotoxic activity in the rabbit ileal loop and the Using chamber assays (Maurelli *et al*., 1998). The inhibitor of the virulence plasmid-encoded *Shigella* enterotoxins proved to be cadaverine, the product of the decarboxylation of lysine. Cadaverine was also found to be responsible for blocking the ability of an LDC-expressing *S. flexneri* to elicit transepithelial migration of polymorphonuclear neutrophils in a polarized tissue culture model system for the inflammatory response (McCormick *et al*., 1999). As attenuation of virulence phenotypes is linked to expression of LDC (and subsequent production of cadaverine) in an *S. flexneri* 2a strain transformed with the *cadA* gene from *E. coli* K-12, *cadA* has the properties of an antivirulence gene for *Shigella*.

Table 1. Effect of *cadA* expression on virulence phenotypes of *Shigella flexneri* 2a

	Wild type	CadA$^+$
HeLa cell invasion	+++	+++
Plaque formation	+++	+++
Apoptosis induction	+++	+++
Fluid secretion ileal loops	0.6 mL cm^{-1}	No fluid
ΔIsc in Ussing chambers*	103.0	33.8
Sereny test	+++	++ (delayed)
PMN transepithelial migration	+++	–

*ΔIsc is the change in short-circuit current in a rabbit intestinal tissue. It is a measure of enterotoxicity.

When the genome of *S. flexneri* 2a was examined, it was discovered that the region corresponding to where *cadA* maps in *E. coli* K-12 had undergone a large deletion: a 'black hole' (Maurelli *et al*., 1998). Sequence analysis of the *cadA* region of four *Shigella* lineages revealed genetic arrangements that are distinct in each strain examined (Day *et al*., 2001). Insertion sequences, a phage genome, and/or loci from different positions on the ancestral *E. coli* chromosome disrupted the *cadA* locus to form distinct genetic linkages unique to each species of *Shigella* (Fig. 1). None of these

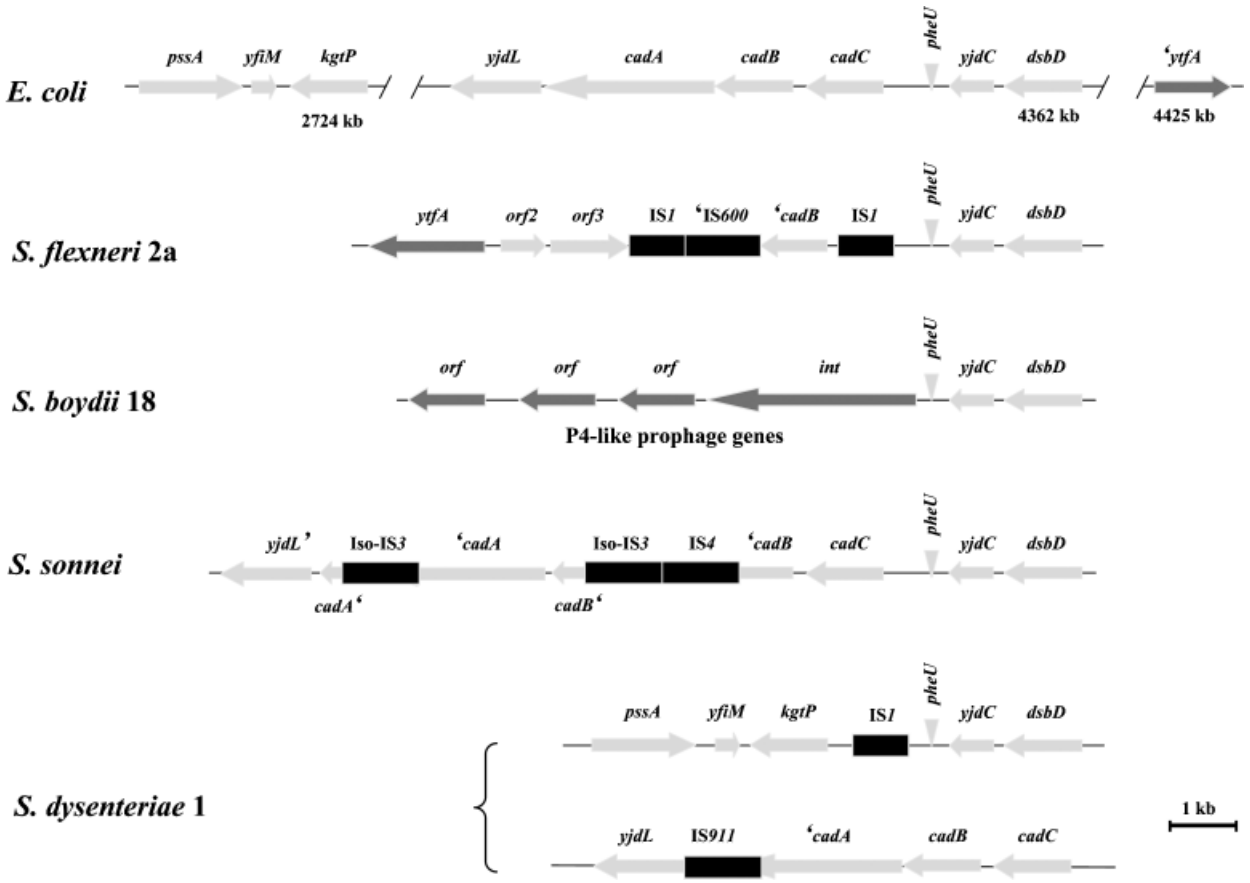

Fig. 1. Novel genetic organization resulting from the *cadA* pathoadaptive mutations in four *Shigella* lineages. Gene loci are depicted as arrows, insertion sequences as black rectangles, and the *pheU* tRNA locus as an inverted triangle; truncated ORFs and insertion sequences are indicated by an apostrophe. The chromosomal maps are aligned at the *yjdC* locus to facilitate comparison. The locations (in kilobase pairs) of *kgtP, dsbD*, and *ytfA* on the *Escherichia coli* K-12 chromosome are indicated below each ORF. The *cad* operon in *Shigella dysenteriae* 1 has translocated and is not linked to *yjdC*. It is located 24 kb clockwise of *pheU* and is depicted below the *pheU* region. Reprinted from (Day *et al*., 2001).

novel gene arrangements were observed in representatives of all *E. coli* phylogenies examined. These observations indicate that inactivation of the *cadA* antivirulence gene occurred independently in each *Shigella* lineage. The convergent evolution of these pathoadaptive mutations demonstrates that, following evolution from commensal *E. coli*, strong pressures in host tissues selected *Shigella* clones with increased fitness and virulence through the loss of an ancestral trait (i.e. LDC).

Additional pathoadaptive mutations and antivirulence genes for *Shigella*

Formal and coworkers identified *kcpA*, a chromosomal gene in *S. flexneri* 2a that is required for the ability of the bacteria to provoke keratoconjunctivitis in a guinea-pig model for *Shigella* virulence (Formal *et al*., 1971). When *S. flexneri* 2a was mated with *E. coli* K-12 and the *kcpA* region was replaced with the homologous region from *E. coli*, the *S. flexneri* transconjugants lost the ability to produce keratoconjunctivitis. The nature of the *kcpA* gene remained a mystery for many years until Nakata *et al*. demonstrated that *kcpA* was not a virulence gene of *S. flexneri* (Nakata *et al*., 1993). The loss of virulence in the transconjugants was due to the transfer of a gene contained in a remnant of a cryptic prophage located in the *E. coli* K-12 chromosome, which, when introduced into the virulent strain of *S. flexneri*, attenuated virulence. The prophage gene, *ompT*, encodes a surface protease that degrades IcsA/VirG, a *Shigella* protein that is required for intra- and intercellular motility and production of keratoconjunctivitis in the guinea-pig. Thus, *ompT* fulfills the definition of an antivirulence gene for *Shigella* as its introduction into *Shigella* attenuates virulence. Further analysis showed that the prophage remnant, designated DLP12, is absent from all lineages of *Shigella*. However, there is no evidence that the DLP12 of *E. coli* K-12 is present in any of the lineages of *E. coli* that are thought to have given rise to *Shigella*. So it can be argued that *ompT* and the absence of DLP12 in *Shigella* spp. do not constitute a true black hole as DLP12 does not appear to have been present in the ancestors of *Shigella*. Nevertheless, *ompT* is an antivirulence gene for *Shigella*.

The absence of curli expression in *Shigella* spp. is a potential example of another pathoadaptive mutation. Curli are thin aggregative surface fibers encoded by *csg* genes that are expressed by strains of *E. coli* and *Salmonella typhimurium*. Curli mediate binding to extracellular matrix proteins, biofilm formation on inert surfaces, and internalization by eucaryotic cells (Olsen *et al*., 1989; Gophna *et al*., 2001). Interestingly, while curli are capable of inducing an inflammatory response in sepsis, invasive strains of bacteria tend not to produce curli. A survey of *csg* genes in all four species of *Shigella* revealed that the *csg* loci are disrupted by insertions or deletions in a wide variety of isolates of diverse serotypes and geographical origin (Sakellaris *et al*., 2000). Isolates of enteroinvasive *E. coli* also contain *csg* loci that are mutated by insertion or deletion. By contrast, curli are expressed by 60% of environmental isolates of *E. coli* (Olsen *et al*., 1993). Taken together, these observations suggest a strong pathoadaptive selective pressure against expression of this surface appendage in *Shigella* and thus present the possibility that curli genes are antivirulence genes. Our laboratory is currently testing this hypothesis.

A 'black hole' in *Burkholderia mallei* and *Burkholderia pseudomallei*

Significant environmental changes that accompany the shift to a pathogenic lifestyle (and corresponding changes in selective pressures) potentially expose antivirulence genes in each newly evolved pathogen. While these loci may be eliminated by pathoadaptive mutations to maximize fitness in the new (pathogenic lifestyle) niche, these mutations may occur at the expense of the bacterium's fitness in the old (nonpathogenic lifestyle) niche. The case of uropathogenic *E. coli* cited earlier is an example of this trade-off. Increased virulence due to mutation of the fimbriae adhesion comes at the cost of reduced fitness in the nonpathologic niche (Sokurenko *et al*., 1998). Likewise, pathogens that exhibit a narrow or novel host or tissue range relative to their nonpathogenic ancestors exhibit characteristics consistent with those predicted to result from elimination of antivirulence genes. Not surprisingly, pathogens that exhibit these characteristics are often more virulent than even their pathogenic relatives. For example, the arginine deiminase cluster that is present in the chromosome of *Bacillus cereus* appears to be entirely deleted from *Bacillus anthracis* (Ivanova *et al*., 2003). The selective pressure for loss of this gene cluster may have been that ammonium production by arginine deiminase is unfavorable for *Bacillus anthracis* as ammonium inhibits receptor-mediated uptake of the anthrax lethal toxin.

Another example can be found in the species *Burkholderia*. *Burkholderia mallei* is an equine and human pathogen that causes a fatal systemic disease through infection of mucosal membranes. It evolved from *Burkholderia pseudomallei*, a soil organism that is capable of causing an opportunistic but localized infection in a number of animal hosts (Woods *et al*., 1999; Woods, 2002). *Burkholderia pseudomallei* is believed to share a common ancestor with *Burkholderia thailandensis*, a nonpathogenic soil saprophyte but *Burkholderia mallei* is not found in soil. Moreover, many other traits associated with *Burkholderia pseudomallei*, such as motility and multi-drug efflux pumps, are not expressed by *Burkholderia mallei*. Comparison of the sequenced genomes of the two organisms did not reveal operons or

biochemical systems unique to *Burkholderia mallei*. Thus, there is no evidence of gain of function acquired through HGT that may increase the virulence and fitness of *Burkholderia mallei* in the host. Rather, the 6.0 Mbp *Burkholderia mallei* genome appears to be a subset of the 7.2 Mbp *Burkholderia pseudomallei*, genome suggesting that the significant increase in virulence and narrowing of host range observed in *Burkholderia mallei* is associated with loss of multiple ancestral loci (i.e. antivirulence genes). This hypothesis awaits testing and specific antivirulence genes in *Burkholderia mallei* have not been identified.

The antivirulence nature of an operon encoding arabinose catabolic enzymes has been demonstrated for *Burkholderia pseudomallei* (Moore *et al.*, 2004). This operon, encoded within *Burkholderia thailandensis*, the nonpathogenic ancestor of *Burkholderia pseudomallei*, is absent from the pathogen's genome. Reintroduction of this operon into *Burkholderia pseudomallei* permits growth on arabinose but significantly reduces virulence in the Golden Syrian hamster model (Moore *et al.*, 2004). The precise nature of the antivirulence effect has not been determined but experimental data suggest that growth of *Burkholderia pseudomallei* expressing the arabinose utilization genes in L-arabinose results in down-regulation of T3SS genes that are required for virulence in hamsters (Moore *et al.*, 2004). While there is not yet any experimental evidence, it is likely that loss of arabinose utilization genes is also a pathoadaptive mutation in *Burkholderia mallei* as it adapted to the more restrictive environment presented to a host-adapted pathogen unable to persist outside of an equine host. Thus, the arabinose utilization locus harbors antivirulence genes for pathogenic *Burkholderia*.

How to find antivirulence genes

Searching for antivirulence genes first requires identification of a closely related nonpathogenic relative of the pathogen being studied. The next step is a comparison of phenotypic traits. Traits that are absent from pathogenic bacteria but that are expressed by their nonpathogenic ancestors may be identified using phenotypic arrays that examine hundreds of phenotypes (and the functionality of thousands of genes encoding these traits) in a single experiment (Bochner *et al.*, 2001). Once a list of phenotypic differences is constructed, a thorough knowledge of the pathogen and a good sense of intuition about which traits could be incompatible with virulence are needed. Needless to say, a robust set of assays for measuring virulence is also required for testing potential antivirulence genes. Identification of previously unrecognized variations in phenotypes expressed by pathogens and their cognate commensal relatives may lead to the discovery of additional pathoadaptive mutations in *Shigella* spp. as well as other bacterial pathogens.

Phenotypes observed in the laboratory represent only a subset of all the potential phenotypes expressed by an organism as thousands of genes in each sequenced bacterial genome encode completely unknown functions. Methods that do not focus on laboratory phenotypes will prove useful for the identification of additional pathoadaptive mutations. These methods will include functional genomic strategies that exploit data provided by genome sequencing studies. For example, comparison of the sequence of a pathogen genome with that of its nonpathogenic ancestor provides a direct means of identifying loci acquired through horizontal gene transfer as well as ancestral genes that have been inactivated and/or deleted from the pathogen genome. Inactivated ancestral genes may represent pathoadaptive mutations. Once again, the challenge will be to determine which of the inactivated genes encodes factors that inhibit fitness in the virulence niche.

Conclusions

Pathogens evolve through the horizontal acquisition of genetic information in the form of plasmids, bacteriophages, or PAIs. Subsequent to this 'quantum leap', selective pressures favor pathoadaptive mutations that result in the loss or inactivation of genes incompatible with the pathogenic lifestyle of the newly evolved pathogen. Thus, optimal fitness in this niche is achieved. *Shigella* spp. represent the index model in which this paradigm of pathogen evolution has been established (Fig. 2).

The observations of antivirulence genes in *Shigella* suggest criteria for the identification of antivirulence genes in other pathogens:

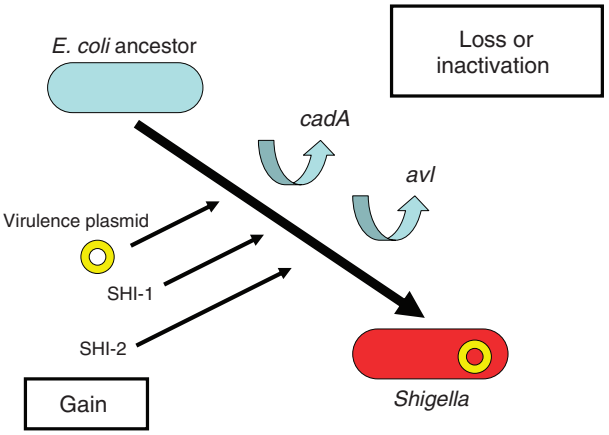

Fig. 2. Model of the evolution of *Shigella* from an ancestral *Escherichia coli*. Horizontal gene transfer and pathoadaptive mutation events are shown. SHI-1 and SHI-2 are the *Shigella* pathogenicity islands located on the chromosome. *cadA* and *avl* represent the genes for lysine decarboxylase and other (as yet unidentified) antivirulence genes, respectively.

(1) The antivirulence gene must be present and expressed in closely related or ancestral species occupying the nonvirulent ancestral niche but absent (or mutated) from pathogenic clones that colonize host tissues; and
(2) Expression of the antivirulence gene by the pathogen in host tissues must attenuate virulence and/or inhibit fitness.

These criteria form a type of converse Koch's postulates and highlight the value of identifying and studying ancestors of pathogenic organisms and their nonpathogenic relatives. Application of the converse Koch's postulates should lead to identification of antivirulence genes lost by pathoadaptive mutation. These genes should provide us with new insights into the ecology and evolution of bacterial pathogens as well as identify new products that can block the activity of virulence factors required for pathogen survival in host tissues. Thus, the study of antivirulence genes holds the potential for discovery of new pathogen-specific therapies and the development of safer, attenuated vaccine strains.

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